Molecular characterization of Vig4/Vrg4 GDP-mannose transporter of the yeast *Saccharomyces cerevisiae*

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Abstract ***Saccharomyces cerevisiae*** **Vig4/Vrg4 protein is a Golgi membrane protein which has multiple transmembrane domains and is essential for transport of GDP-mannose across the Golgi membrane. By immunoprecipitation of detergent-solubilized tagged protein, we found that this protein exists as oligomer. Two mutants *vig4-1* and *vig4-2* had amino acid substitutions in the C-terminal region, Ala286Val and Ser278Cys, respectively. In accord with these mutations, trimming of the C-terminal hydrophobic part close to the region impaired the function and traffic of the proteins from the endoplasmic reticulum to the Golgi compartments.**

Key words: Golgi membrane; GDP-mannose transporter; Yeast; *VRG4/VIG4*; *Saccharomyces cerevisiae*

1. Introduction

In eukaryotes, many polypeptides are modified with glycosyl residues which contribute to their stability and function. *N*-glycosylation on suitable asparagine and *O*-glycosylation on serine or threonine occurs during the translocation of the polypeptides in the endoplasmic reticulum (ER) and traffic in the Golgi compartments. In *Saccharomyces cerevisiae*, mannose is the sole sugar added on protein except two *N*-acetylglucosamine (GlcNAc) residues which link each *N*-glycan to asparagine. The linkage between the polypeptide and sugar is made in the ER using dolichol intermediates and then elongation of mannose moiety occurs in the Golgi using GDP-mannose [1–3]. GDP-mannose is synthesized in the cytosol by Vig9 GDP-mannose pyrophosphorylase [4]. It should be transported across the Golgi membrane into the lumen by a specific nucleotide sugar transporter which also functions as an antiporter of GMP back into the cytoplasm [5].

Among 217 recessive *vig* **v**anadate resistance and **i**mmature **g**lycosylation mutants of *S. cerevisiae* which we isolated and classified in 9 complementation groups [4], 16 belonged to *vig4*. Genetic and sequence analysis revealed that it was allelic to the mutation named *vrg4* [6,7] or *van2* [8] which were similarly enriched by vanadate-resistance [6]. Both *N*- and *O*-glycosylation in the Golgi was severely affected in these mutants and the null mutant was lethal. Dean and colleagues have reported that *VRG4* encodes an essential Golgi membrane protein containing multiple membrane-spanning domains [7] and is required for GDP-mannose transport into the lumen of the Golgi by in vitro analyses [9].

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Addition of mannose by various mannosyltransferases is an elaborative function of the Golgi and the supply of donor nucleotide sugar, GDP-mannose, is the prerequisite. So, GDP-mannose/GMP transporter should be the essential component for the function of this organelle. As the *vrg4-2* mutant cell had a disordered endomembrane system [7] and *VIG4*-overexpressing cell developed multiple lamellar structure similar to the Golgi apparatus of mammalian secretory cells (our unpublished result), Vrg4/Vig4 protein might concern in the architecture of the organelle. In this paper, we report that Vig4/Vrg4 protein is oligomeric and its C-terminal region is important for the function and conformation of this molecule.

2. Materials and methods

2.1. Strains and media

Escherichia coli DH5α (F$^-$ ϕ80*lacZ*Δ*M15*, *supE44* Δ*lacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used in plasmid construction. *S. cerevisiae* KA31-1A (*MATa* Δ*ura3* Δ*trp1* Δ*his3* Δ*leu2*) [10], K2 (*MATa vig4-1 leu1*) and C4 (*MATα vig4-2 lys1*) were used.

2.2. Plasmid construction

The DNA fragments encoding the full-length Vig4, C-terminal truncated Vig4Δ12, Vig4Δ35 and Vig4Δ62 were prepared by polymerase chain reaction using sense primer (nucleotide from −968 to −943; A of initiation ATG was defined as nucleotide 1) and antisense primers (nucleotide 996–1031, 957–976, 884–902 and 823–841, respectively). Primers were designed to place *Bam*HI site at the 5′-end and *Xho*I site at the 3′-end for cloning in pCH1 (2 μm, *URA3*) to construct C-terminal fusion with MYC hexamer (Vig4-MYC) [10] or pYN116 (2 μm, *URA3*) to construct C-terminal fusion with HA trimer (Vig4-HA). The expression units were cut out and subcloned in pRS314 (*CEN6*, *TRP1*) or pRS316 (*CEN6*, *URA3*) [11], if necessary.

2.3. Immunological methods

Immunoprecipitation of tagged protein, immunoblotting and indirect immunofluorescence microscopy were done as previously described [10]. Disintegration of the cell was done by shaking with glass beads instead of zymolyase digestion. Unbroken cells were removed by centrifugation at 2000×*g* for 5 min, before addition of detergent, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS, Sigma). In some experiments, the supernatant after centrifugation at 10000×*g* for 20 min (S10) was used to exclude large membranous materials.

3. Results and discussion

3.1. Oligomerization of Vig4 protein

Some membrane transporters are reported to form homodimer and leucine-zipper motifs were suggested to be involved in oligomerization [5]. Though a leucine-zipper motif was not found in Vrg4/Vig4 protein, we examined whether the functional form of Vig4 is monomeric or oligomeric. The genes encoding epitope-tagged derivatives, Vig4-MYC and Vig4-HA, were constructed. The *vig4* mutants show increased

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drug sensitivity and decreased protein glycosylation (Poster and Dean [7] and our unpublished results) as a result of defective transport of GDP-mannose in the Golgi complex [9]. The MYC hexamer or HA trimer tags at the C-terminus did not impair the function of Vig4 because the defect of the *vig4-1* mutant was rescued by introduction of each plasmid (Fig. 3).

Transformant cells were broken with glass beads, unbroken cells and debris were removed by low-speed centrifugation (2000×*g*, 5 min) and the membrane proteins were solubilized with 0.5% CHAPS. After high-speed centrifugation (100 000×*g*, 1 h) to remove insoluble materials, the MYC-tagged protein was collected by immunoprecipitation, and subjected to SDS-PAGE and immunoblotting. Fig. 1A shows that Vig4-HA protein was precipitated by anti-MYC antibody if both proteins were expressed in the same cells (lane 3), while it was not precipitated if only Vig4-MYC (lane 1) or Vig4-HA (lane 2) was present in the cell. Thus, Vig4-MYC and Vig4-HA has physical interaction. The association should be intrinsic and occur in vivo, because coprecipitation was not observed when the detergent extracts were mixed in vitro (lane 4). Experiments using HA for immunoprecipitation and MYC for immunoblotting gave similar results (data not shown).

The specificity was further supported by the fact that Vig4-HA was not coprecipitated with other Golgi type II membrane proteins, Mnn9-MYC or Sed5-MYC, in similar conditions (data not shown). Recovery of Vig4-HA in the precipitate increased if the amount of Vig4-MYC increased (Fig. 1B). It was much reduced if Triton X-100 was used instead of CHAPS (data not shown). Triton X-100 may interfere the protein-protein interaction more extensively. This may suggest some kinds of hydrophobic interaction play a role in oligomer formation.

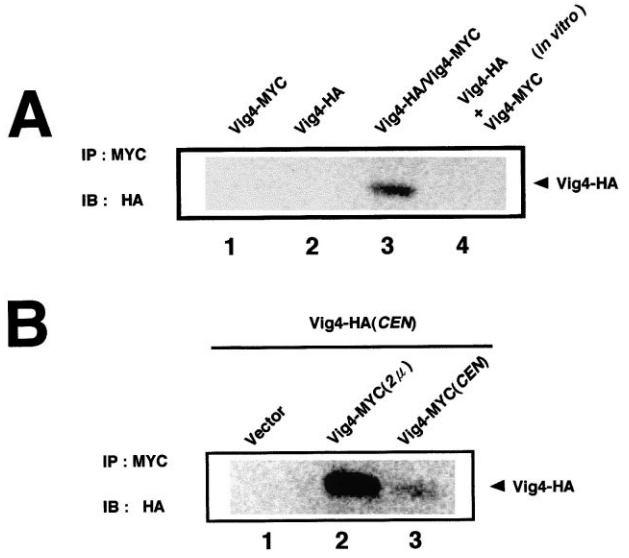

Fig. 1. Co-immunoprecipitation of epitope-tagged Vig4 protein after solubilization by the detergent CHAPS. Cells were broken by glass beads and membrane proteins were solubilized by 0.5% CHAPS. After centrifugation at 100 000×*g* for 1 h, the supernatant was applied to immunoprecipitation by anti-MYC monoclonal IgG 9E10. Immunoprecipitates were analyzed by immunoblotting by anti-HA monoclonal IgG 12A5 and horseradish peroxidase-conjugated goat anti-mouse IgG and ECL detection system (Amersham). A: Detergent extracts were prepared from cells expressing Vig4-MYC (lane 1), Vig4-HA (lane 2), or both (lane 3). Detergent extracts containing Vig4-MYC and Vig4-HA were prepared separately and mixed on ice and kept for 30 min before immunoprecipitation (lane 4). B: Vig4-HA was produced from *CEN* plasmid in combination with 2 μm vector (lane 1), 2 μm plasmid expressing Vig4-MYC (lane 2), or *CEN* plasmid expressing Vig4-MYC (lane 3).

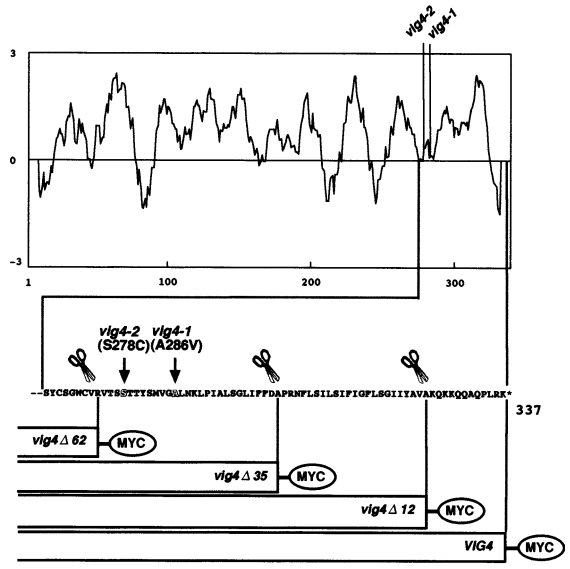

Fig. 2. The point and deletion mutants and hydropathy profiles of Vig4 protein. Hydropathy was plotted according to Kyte and Doolittle [12] at a window of 15 amino acids. The positions of the amino acid substitutions, deletions and MYC-tagging were shown schematically.

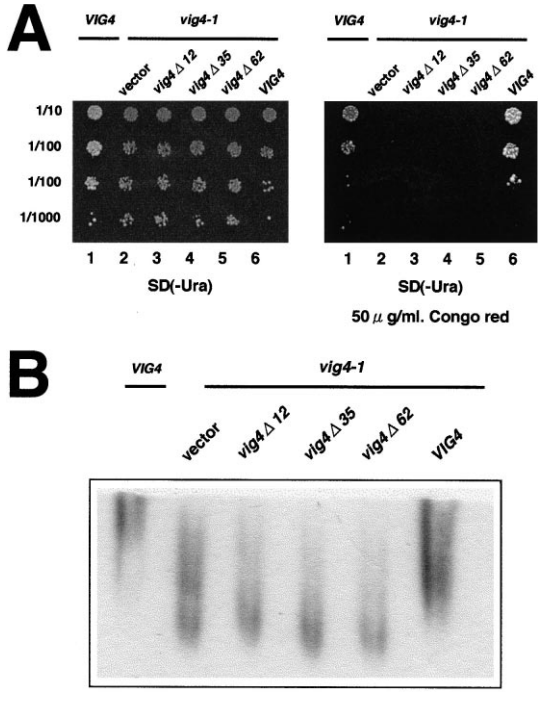

Fig. 3. Activities of the deletion derivatives as assayed by rescue of the *vig4-1* mutant phenotypes. A: Congo red sensitivity was assayed by growth of serially diluted fresh cultures spotted on the agar plates with 0 or 50 μg/ml of Congo red; (1) KA31-1A, (2) *vig4-1*/pCH1, (3) *vig4-1*/pCH1-Vig4Δ12, (4) *vig4-1*/pCH1-Vig4Δ35, (5) *vig4-1*/pCH1-Vig4Δ62 and (6) *vig4-1*/pCH1-Vig4. B: Activity staining of invertase in 7.5% SDS-PAGE of the periplasmic fractions of the wild-type KA31-1A and *vig4-1* transformants.

3.2. Sequence alterations in vig4 mutants

To find functionally important amino acids in Vig4, we determined the nucleotide sequences of our mutant alleles. In *vig4-1*, the 856th cytosine was replaced with thymine and accordingly the 286th amino acid alanine was replaced with valine. In *vig4-2*, the 833rd cytosine was replaced with guanine and accordingly the 278th serine was replaced with cysteine. These altered amino acids were only 10 amino acid apart in a relatively less hydrophobic region between the C-terminal hydrophobic peaks [12] (Fig. 2).

3.3. Effect of truncation from the C-terminus.

As the point mutations were near the C-terminus, we constructed three short deletions of 12, 35 and 62 amino acids (Fig. 2) to see the role of the C-terminal region. As shown in Fig. 3, even the shortest deletion which removed only the hydrophilic stretch of 12 amino acids (Vig4Δ12) lost the activity to rescue the defects of *vig4-1* mutation; sensitivity to Congo red and decreased glycosylation of invertase. Other deletions were also not functional in these respects (Fig. 3).

Glutathione *S*-transferase or green fluorescent protein could be fused to the C-terminus of Vig4 without large loss of activity (data not shown). The C-terminal 12 amino acids of Vig4 are hydrophilic, containing 4 glutamines, 4 lysines and 1 arginine. The positive charge of this peptide may play a role for its activity.

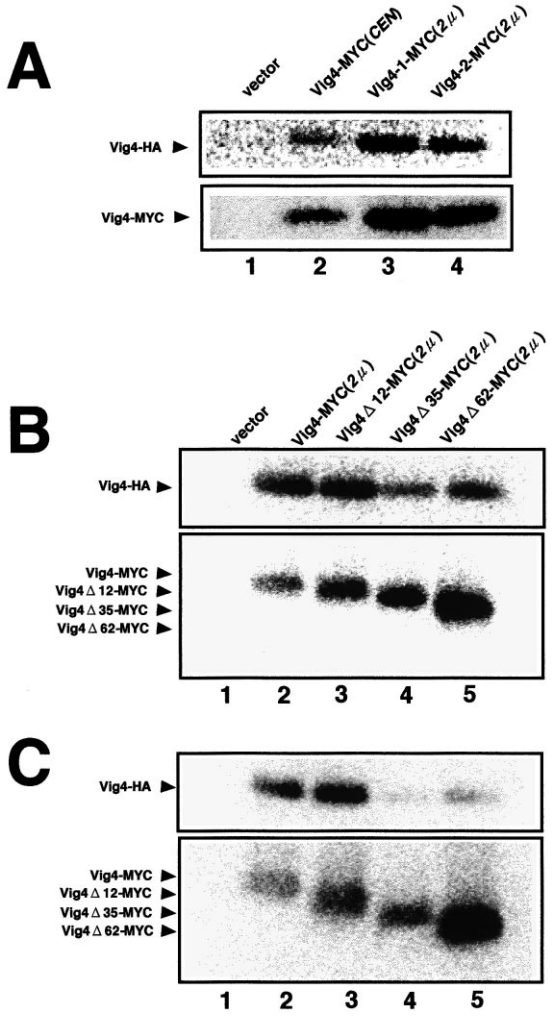

Fig. 4. Coprecipitation of the wild-type Vig4-HA with MYC-tagged mutant derivatives. A: In KA31-1A, Vig4-HA was produced from *CEN* plasmid in combination with 2 μm vector (lane 1), *CEN* plasmid expressing Vig4-MYC (lane 2), 2 μm plasmid expressing Vig4-1-MYC (lane 3) or 2 μm plasmid expressing Vig4-2-MYC (lane 4). Immunoprecipitation was done with anti-MYC antibody, and immunoblotting was done with anti-HA (upper) or anti-MYC (lower) antibodies. The detergent extract was prepared from supernatant of $2000\times g$, 5 min centrifugation (Total lysate). B: Similar immunoprecipitation was done as in (A). The precipitation probe was none (lane 1), Vig4-MYC (lane 2), Vig4Δ12-MYC (lane 3), Vig4Δ35-MYC (lane 4) or Vig4Δ62-MYC (lane 5). C: Same as (B) except the detergent extract was prepared from the supernatant of $10\,000\times g$, 20 min centrifugation (S10).

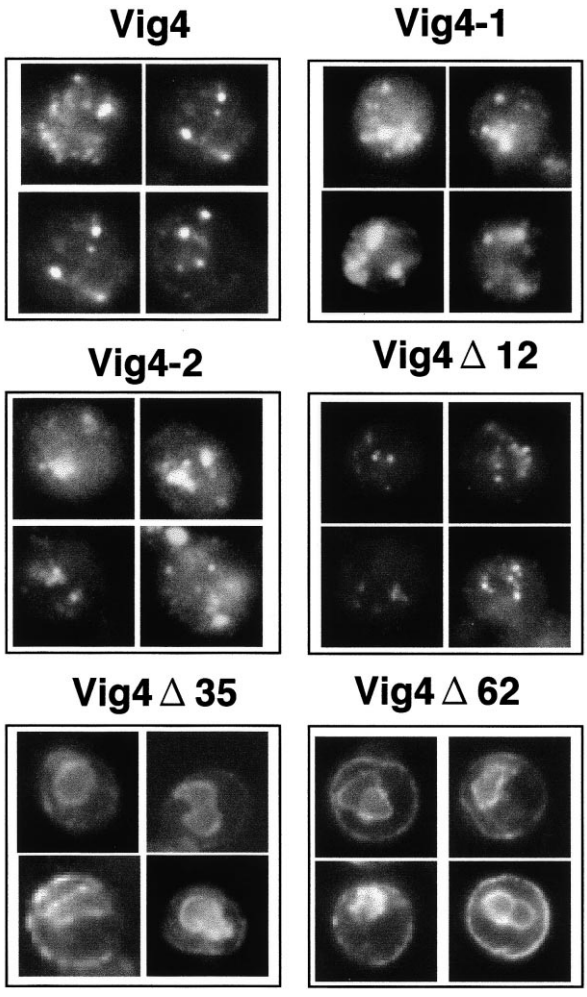

Fig. 5. Intracellular localization of Vig4 and the mutant proteins visualized by immunofluorescent staining. Transformant cells which produce various MYC-tagged derivatives of Vig4 were stained with anti-MYC 9E10 and fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG and photographed with a BH2-RFC microscope (Olympus, Tokyo).

3.4. Oligomer formation of the mutant Vig4 derivatives

As we have shown that the wild-type Vig4 protein forms oligomers, we next examined whether the point and deletion mutant proteins could join in the oligomer or not. We constructed transformants in which the mutant Vig4 polypeptides were tagged with MYC epitope and the wild-type Vig4 was tagged with HA epitope. The MYC-tagged mutant protein was collected from CHAPS-solubilized lysate by anti-MYC

antibody and then existence of the wild-type Vig4-HA in the precipitate was examined by immunoblotting. As shown in Fig. 4A, Both of the two MYC-tagged point mutant proteins joined in the oligomeric form with the wild-type Vig4-HA protein. Three truncated proteins also coprecipitated the HA-tagged wild-type protein (Fig. 4B). These results suggest the C-terminal 12 amino acids are not essential for oligomer formation. When we used the supernatant of $10\,000 \times g$ centrifugation (S10) instead of total lysate, Vig4Δ35-MYC and Vig47Δ62-MYC were not able to coprecipitate the wild-type protein (Fig. 4C). This suggests these deletion derivatives were on rapidly sedimenting membranes which were removed in S10.

3.5. Localization of the mutant proteins

Intracellular localization of the MYC-tagged mutant Vig4 derivatives were examined by immunofluorescent staining. As shown in Fig. 5, Vig4-1, Vig4-2 and Vig4Δ12 protein gave punctate staining patterns indistinguishable from the wild-type protein. This suggests all these proteins were mainly localized in the Golgi compartment. On the other hand, Vig4Δ35 and Vig4Δ62 gave a typical image comprised of central ring and threads along the contour of the cell which indicated that the proteins were in the ER. The localization coincided with the rapid sedimentation of the proteins. This suggests the C-terminal hydrophobic stretch plays a role in the traffic of Vig4 protein from the ER to the Golgi.

Although the topology should be determined precisely, we might expect the region Ser278–Ala286 is a candidate of the site of interaction with the transport substrates GDP-mannose and GMP [5,9]. Crosslinking with the photoreactive analogs of GDP-mannose should be useful to examine this issue. Site-directed mutagenesis of these regions might also help to determine the essential residues for transport of substrates.

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